Nanosize Bioconjugates

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pH-Responsive Nutraceutical–Mesoporous Silica Nanoconjugates with Enhanced Colloidal Stability**

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Among recent discoveries in the area of nanomaterials, mesoporous silica nanoparticles (MSNs) have emerged as one of the most promising carriers for controlled and targeted drug delivery.[1] The application prospect of MSNs in nanomedicine arises from their outstanding porosity features, facile synthesis, tuneable pore and particle size, versatile inner and outer surface chemistry, and capacity to load and unload various cargos ranging from relatively small molecules, e.g., ibuprofen,[2] to very large ones, e.g., siRNA.[3] Additionally, the high in vitro and in vivo biocompatibility of MSN-based systems and the possibility of precise control over adsorption and release kinetics, have positioned them as leading candidates for next generation therapeutic carriers.[1f,g,4] Nevertheless, the key challenge remains to simultaneously achieve precise targeting and higher colloidal stability, especially in physiological media.[5] To reach these objectives, several delivery systems have recently been proposed on the basis of various stimuli, that is, pH,[6] light,[6e–g] temperature,[6,7] chemical reactions,[8] enzymes,[8,9] and so forth. Amongst these, targeted release based on pH variations has received the greatest attention. For instance, in the case of cancer therapy, the pH difference between normal cells and cancer cells can be used for targeting tumor cells.[6,10] Furthermore, pH in the human body naturally varies between the different organs and tissues making the pH-triggered approach one of the most efficient strategies for oral drug delivery.[11] These pH differences have been smartly used to synthesize "targeted" pH-responsive systems using MSNs.[6h,12] However, one limitation of these functionalized MSN-based systems is their low physiological and colloidal stability, a characteristic being rarely discussed in the literature despite its crucial importance.[1a] Evidently, the design of pH-responsive MSNs possessing enhanced stability as well as non-toxic/natural pore "gating" remains a complex and exciting challenge. In parallel, over the last few years, the use of proteins as stimuli-responsive gating systems has arisen as an efficient alternative to classical biopolymers.[9a,13,14] Remarkably, these readily available low-cost materials could offer considerable advantages owing to their natural biocompatibility and biodegradability. Moreover, abundant reactive groups (e.g., lysine groups), which are present in the proteins, can be used for grafting, cross-linking or other chemical modifications.[13a,e,14a,b] In particular, Caillard et al. reported that tablets prepared from succinylated β-lactoglobulin (nutraceutical) exhibited excellent properties as a controlled drug delivery excipient.[14a] Indeed, pronounced conformation changes of this protein in acidic media limited erosion and swelling at stomach pH, whereas higher pH (6.8–7.5) led to the opposite effect. In turn, this resulted in very low drug release in simulated gastric fluid (SGF, pH 1.2) but high drug release in simulated intestine fluid (SIF, pH 7.4).[14a,b] More importantly, the succinylated β-lactoglobulin acted as emulsifier and pH buffer inside the tablet, avoiding drug alteration.[14a,b] Therefore, these unique features make this protein an exciting biomaterial for the protection and intestinal release of gastro-sensitive compounds.

To synergistically combine both the advantages of MSNs and β-lactoglobulin, we report here the first example of binding of succinylated β-lactoglobulin onto functionalized MSNs exhibiting a 3-D pore network (*Ia3d*) and evaluate the potential of the resulting bioconjugate as a new pH-responsive oral drug delivery system with high colloidal stability and low toxicity. For this study, we chose ibuprofen and acridine as hydrophobic and hydrophilic model drug candidates, respectively. The nutraceutical–MSNs conjugate system is expected to limit premature release and/or denaturation of the drug/dye in the stomach (pH 1.2) and allows for a controlled release of drug/dye molecules in the intestine (pH 6.8–7.4).

As illustrated in Scheme 1, we first synthesized mesoporous MCM-48 nanoparticles (MNps)[15] which were then functionalized using 3-aminopropyl triethoxysilane (APTES) to yield NH_2-MNps. These particles were subsequently loaded with a model drug or dye, i.e., ibuprofen (IBU) or acridine (ACR), respectively. The particles were further placed in

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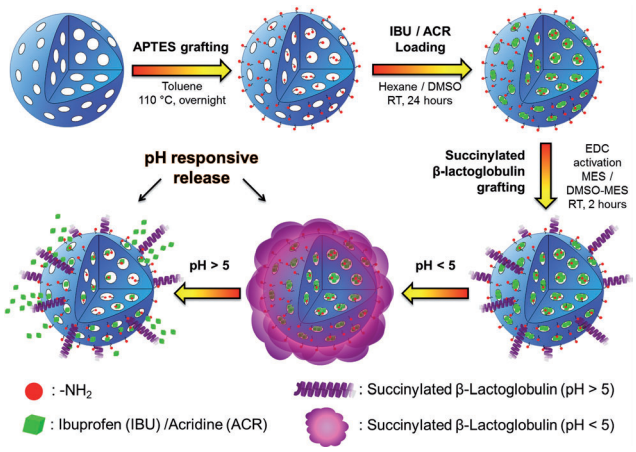

Scheme 1. Schematic representation of the post-grafting, bio-functionalization, and pH-responsive release of β-drug/dye-MNps.

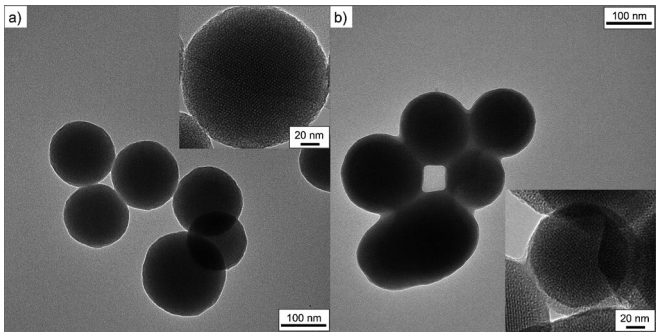

Figure 1. TEM images of a) NH_2-MNps and b) β-MNps. Insets show HRTEM images of both materials.

a dimethyl sulfoxide/2-(*N*-morpholino)ethanesulfonic acid (DMSO/MES) or MES 0.1 M buffer (pH 6) in presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) followed by the addition of negatively charged succinylated β-lactoglobulin.[16] The reaction proceeded for 2 h leading to covalent attachment of the protein through bonding between the amino groups and the carboxylic acid groups of the protein (synthesis details can be found in Supporting Information and Scheme S1),[17] which afforded the final pH-responsive β-drug/dye-MNps complex. Typically, at $pH < 5$, succinylated β-lactoglobulin undergoes a gelation process that has pronounced effect on its secondary structure, leading to the formation of intermolecular hydrogen-bonding β-sheet.[14,18] This conformational change is associated with lower solubility of the succinylated protein in acidic media owing to abundant carboxylic acid groups,[13a,14] resulting in a pH dependent gel "shell" around the MNps. This gel will prevent the drug/dye molecule release from the mesopores. At pH above the isoelectric point of the system ($pH > 5$, Figure S1) and at physiological pH, the succinylated β-lactoglobulin remains permeable, allowing the drug/dye to diffuse out (Scheme 1).

Transmission electron microscopy images (TEM) of pristine MCM-48 nanospheres show non-aggregated, well-defined and uniform spherical particles, with an average particle size of ca. 140 nm (Figure S2). The pore network of MNps exhibits excellent mesoscopic order with pore structure being commensurate with the cubic *Ia3d* symmetry, as also confirmed by low-angle X-ray diffraction (XRD) (Figure S3a). N_2 sorption data revealed a typical type IV isotherm with steep capillary condensation step characteristic of high quality material with uniform mesopore size (Figure S4). No changes in particle shape were observed by TEM (Figure 1 a) for the amino-modified MNps (NH_2-MNps) which remained non-aggregated and homogeneous in diameter. However, as expected, a marked decrease in pore volume, specific surface area and pore size was observed for NH_2-MNps compared to pristine MNps (see Table S1, Figure S4), which is in good agreement with the introduction of amines inside the mesopores.[19a] This latter decrease became even more pronounced once drug/dye loading was performed. In addition, one can note the important decrease in the X-ray diffraction intensity which can be correlated to the decrease in the electron density contrast between the silica walls and the drug/dye-loaded mesopores (Table S1, Figures S3, S4).[19b]

After protein grafting, the functionalized drug/dye-loaded systems (denoted β-IBU-MNps and β-ACR-MNps for ibuprofen- and acridine-loaded systems, respectively) exhibited almost no porosity according to N_2 sorption data, which give a first indication of successful grafting of the succinylated protein onto loaded NH_2-MNps (Table S1, Figures S3 and S4). Fourier-transform infrared (FTIR) and thermogravimetric analysis (TGA) data further attested the grafting (Figures S5 and S6, respectively). This conclusion is corroborated by the ^{13}C CP NMR results, presented for ACR-MNps in Figure S7. Indeed, for NH_2-MNps, the three typical chemical shifts ($\delta = 10$, 22, and 43 ppm) characteristic of the aminopropyl chain were observed. After ACR-loading, a wide band located in the range $\delta = 120$–140 ppm appears, related to the aromatic carbons of the dye. Finally, after protein grafting, a strong band located at $\delta = 173$ ppm was detected which substantiates the amide link between the ACR-MNps and the succinylated protein. Moreover, the apparition of bands at $\delta = 25$, 30 and 53 ppm combined with the decrease of the bands related to the aminopropyl group ascertain successful grafting of the protein. Representative TEM and HRTEM images of β-MNps further show that the protein component is coated around and at the interface of the MNps (Figure 1 b). However, it was not possible to confirm the average thickness of the β-lactoglobulin coating.

The stability of NH_2-MNps and β-MNps was monitored by dynamic light scattering (DLS) at physiological pH (7.4) (Figure S8). Hydrodynamic diameter of the particles and aggregation phenomena can substantially affect the properties of such systems, especially when they are designed for cellular drug release, oral delivery and bio-imaging applications.[1a] As exemplified by the correlogram shown in Figure S8a, NH_2-MNps are not stable in phosphate-buffered saline (PBS) at pH 7.4 which is in line with previous reports.[1a] Consequently, no relevant hydrodynamic diameter distribution curve could be obtained due to the formation of very large aggregates. Also, the zeta-potential of NH_2-MNps analysis revealed a weakly positive value, that is, 13 mV, at physiological pH, confirming their electrokinetic instability (Figure S1). Differently, the zeta-potential of β-MNps at

pH 7.4 was found to be −27 mV, suggesting an adequate electrostatic stability. The DLS correlogram of β-MNps in PBS (Figure S8b) demonstrates a satisfactory correlation coefficient and a steep decay indicative of almost monodispersity of these modified particles in suspension. From the calculated hydrodynamic diameter distribution (Figure S8c), one can observe a single peak centered at 190 nm obtained in intensity mode, confirming the very low polydispersity. In addition, a good polydispersity index (PDI) of 0.17 was obtained in PBS (see Table S2). Moreover, the hydrodynamic diameter obtained by DLS in number mode is in good agreement with our TEM observations, taking the hydration corona into account. Colloidal stability of the β-MNps was further probed over 48 h in PBS (see Table S2). One can note a small increase in the hydrodynamic diameter and in the PDI as compared to freshly dispersed particles, but it remains that β-MNps are colloidally stable after two days in physiological media. This excellent result evidences that the succinylated β-lactoglobulin "coating" enhances drastically the colloidal stability of MSNs, which is in line with the foaming and emulsifying properties of this protein.[14a] In addition, since β-lactoglobulin is biocompatible and biodegradable, it can be viewed as a very attractive and efficient alternative to stabilize MSN suspensions.

To study the pH-dependent gating effect, gelation properties of the β-MNps were also monitored. As discussed above, at $pH < 5$, succinylated β-lactoglobulin can form a gel-like film covering the surface of the mesoporous nanoparticles. In contrast, at $pH > 5$, the solubility of the succinylated β-lactoglobulin is substantially enhanced resulting in an improved colloidal stability. The pH-dependent gelation of the modified particles was clearly observed at gastric and intestine pH, 1.2 and 7.4, respectively, as shown in Figure S9. A transparent, homogeneous suspension is visible at pH 7.4 (Figure S9a), being typical of β-MNps suspended in PBS, with excellent colloidal stability, as discussed above. In contrast, when β-MNps were suspended in pH 1.2, a gel quickly formed at the bottom of the tube (see Figure S9b). This gel remained insoluble confirming the reported observations of the β-lactoglobulin.[14a]

As this novel system is designed for oral drug delivery applications, a careful verification of the colloidal stability was also performed after 2 h at pH 1.2, in order to insure that the gelation during the gastric emptying does not alter the β-MNp properties observed at pH 7.4 (Figure S10). Indeed, after 2 h in gastric pH solution, β-MNps exhibited the typical correlogram of gelified particles, that is, poor correlation factor indicative of colloidal instability and formation of aggregates. After centrifugation and re-suspension of the same β-MNps in PBS at pH 7.4, a substantial improvement of the colloidal stability was observed. Moreover, a similar hydrodynamic diameter distribution (in intensity mode, that is, a representation that facilitates the observation of large aggregates) was obtained as compared to β-MNps that were freshly suspended in PBS (Figure S10a,b), evidencing the efficiency of the succinylated β-lactoglobulin "coating", even following regular gastric conditions in terms of time and pH. It should be noted that, if the exchange of the solution medium is repeated once or twice more, a progressive loss in the colloidal stability was observed (Figure S10c,d), suggesting a progressive denaturation and degradation of the protein. Such observation agrees with the irreversible conformation change of the succinylated β-lactoglobulin which is known to occur at acidic pH.[14] Though, as reversibility of the system is not expected for oral drug delivery, this progressive biodegradation process may be viewed as an advantage in terms of biocompatibility.

For drug delivery studies, first, the pH-dependent release profile of β-IBU-MNps was investigated at pH 1.2, 5.0 and 7.4 (PBS) as a function of time. Figure 2 depicts the release of

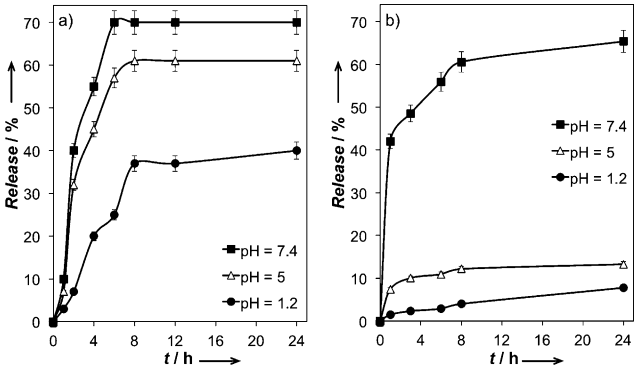

Figure 2. Release of ibuprofen at different pH values from: a) uncoated IBU-MNps, b) coated β-IBU-MNps.

ibuprofen from uncoated and β-lactoglobulin-coated MNps at different pH values. It is evident from Figure 2a that the release of ibuprofen from IBU-MNps shows the so-called burst effect followed by sustained release up to 24 h, releasing about 60% and 70% of the drug at pH 5 and 7.4, respectively. However, at pH 1.2, due to electrostatic and ionic interactions between amino moieties and ibuprofen,[19b] and also taking into account ibuprofen's limited solubility at this pH, only 40% release was achieved. On the other hand, for the succinylated β-lactoglobulin-coated particles, a pH-responsive gating was clearly observed (Figure 2b). Here, only 7.5% and 13% release were attained at pH 1.2 and 5, respectively. Most importantly, β-IBU-MNps released 70% of the drug at the desired physiological pH (7.4), improving significantly the potential for site specificity. Preliminary release kinetic studies were performed in the case of β-IBU-MNps (Table S3), which corroborated the barrier role of the β-lactoglobulin coating. Our release results combined with the observed gelation behavior and the colloidal stability enhancement demonstrate the promising potential of the β-MNp system for the release of gastro-sensitive drugs (no premature release in acidic media avoiding poor bioavailability, undesired side effects and drug denaturation). In addition, a release study at pH 1.2 was also performed using a hydrophilic dye as a cargo, that is, acridine-loaded β-MNps. ACR release at pH 1.2 was significantly lowered in the case of β-ACR-MNps (Figure S11). After 2 h (average gastric transit), only 5.5% ACR release was observed from β-ACR-MNps, whereas 23.8% of ACR was released from uncoated particles (ACR-MNps), that is, a 4-time lower

release in acidic media. As ACR is not soluble in basic pH, no release tests were performed at pH 7.4.

Finally, to assess the biocompatibility of the proposed nanoparticle system, in vitro cytotoxicity was evaluated using pristine MNps, NH_2-MNps and β-MNps on HEK 293 cell lines using an MTS assay (Figure 3). The results demonstrate

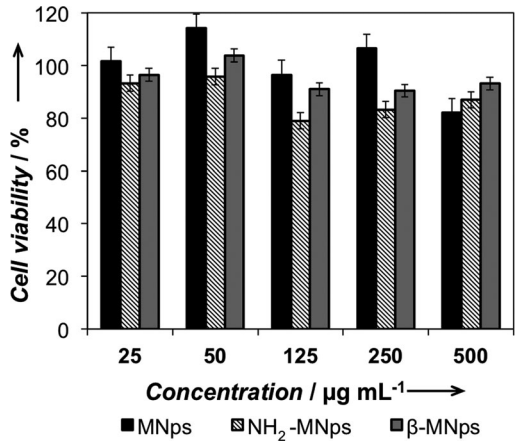

Figure 3. MTS cell viability assay of MNps (black), NH_2-MNps (shaded), and β-MNps (gray) on HEK 293 cells at different concentrations (see Supporting Information for experimental details).

that cytotoxicity of the particles is minimal except at a concentration of 500 $\mu g\,mL^{-1}$. Importantly, at concentrations between 25–250 $\mu g\,mL^{-1}$ NH_2-MNps always showed higher cytotoxicity compared to MNps and β-MNps. However, the cytotoxicity of NH_2-MNps is reduced by the attachment of succinylated β-lactoglobulin on the surface. Consequently, β-MNps showed the highest biocompatibility, which is comparable to classical MNps even at the highest concentration assessed (500 $\mu g\,mL^{-1}$). Such results indicate desirable biocompatibility of the system using the HEK 293 cell model.

In conclusion, we have developed a novel strategy to prepare nutraceutical (β-lactoglobulin)–MSN nanoconjugates with high biocompatibility, pH-responsive properties, and excellent colloidal stability. The attachment of this bioprotein not only protects the drug/dye from leaching in acidic environment (pH 1.2) but effectively allows the release of hydrophobic drugs in desired more basic sites (pH 7.4), while maintaining adequate colloidal stability. The β-MNps remain highly stable in pH 7.4 buffer for over two days, which makes them excellent carriers for sustained-release drug delivery. In vitro biocompatibility tests with HEK 293 cells showed that, the β-MNps exhibit improved biocompatibility compared to traditional amino-functionalized MSNs, irrespective of the concentration. Studies are ongoing to further optimize the gating behavior for delivery or protection of drugs under different pH conditions in order to develop novel smart theranostic carriers.

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